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DECLARATION

I, Makoto AIHARA, Patent Attorney, of SIKs & Co., 8th Floor, Kyobashi-Nisshoku Building, 8-7, Kyobashi 1-chome, Chuo-ku, Tokyo 104-0031 JAPAN hereby declare that I am the translator of the certified official copy of the documents in respect of an application for a patent filed in Japan on August 25, 2000 under Patent Application No. 255402/2000 and that the following is a true and correct translation to the best of my knowledge and belief.

Dated: October 27, 2003

Makoto AIHARA

PATENT OFFICE

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This is to certify that the annexed is a true copy of the following application as filed with this office.

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Applicant(s) : RIKEN
Yoshihide HAYASHIZAKI

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[Inventor]	
[Domicile or Residence]	22-1-201, Inarimae, Tukuba-shi, Ibaraki
[Name]	Yoshihide HAYASHIZAKI
[Applicant for Patent]	
[Identification Number]	000006792
[Name]	RIKEN
[Applicant for Patent]	
[Identification Number]	597157174
[Name]	Yoshihide HAYASHIZAKI
[Administrator]	
[Identification Number]	100092635
[Patent Attorney]	
[Name]	Hisao SHIOZAWA
[Subadministrator]	
[Identification Number]	100096219
[Patent Attorney]	
[Name]	Masazumi IMAMURA
[Subadministrator]	
[Identification Number]	100095843
[Patent Attorney]	
[Name]	Junji KAMATA
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[Document] Specification

[Title of the Invention] Method of Preparing Normalized / Subtracted cDNA

[Claims]

[Claim 1] A method of preparing normalized and/or subtracted cDNAs characterized in that the normalized and/or subtracted cDNA is reverse transcript of mRNA in the form of uncloned cDNA (hereinafter referred to as cDNA tester).

[Claim 2] A method of preparing normalized and/or subtracted cDNAs characterized by comprising the steps of:

I) preparing cDNA testers;

II) preparing polynucleotides (hereinafter referred to as drivers) for normalization and/or subtraction;

III) conducting normalization and/or subtraction and removing tester/driver hybrids and non-hybridized polynucleotide drivers;

IV) recovering the normalized and/or subtracted cDNA.

[Claim 3] The method according to claim 1, wherein said cDNA tester is single strand or double strand.

[Claim 4] The method according to claim 1, wherein said cDNA tester is single strand and said method further compriese a step V) of preparing a second strand of recovered cDNA and performing cloning.

[Claim 5] The method according to any of claims 1-3, wherein in step III), normalization is comprised first, and subtraction is subsequently comprised.

[Claim 6] The method according to any of claims 1-3, wherein in step III), subtraction is comprised first, and normalization is subsequently comprised.

[Claim 7] The method accoring to any of claims 1-3, wherein in step III), said tester and drivers are mixed together and normalization and subtraction are conducted in a single step.

[Claim 8] The method according to any of claims 1-6, wherein said normalized and/or subtracted cDNA is long-strand, full-coding, and/or full-length cDNA.

[Claim 9] The method according to any of claims 1-7, wherein step III) comprises the addition of an enzyme capable of cleaving single-strand RNA driver nonspecifically

bound to single strand cDNA and the removal of said cleaved single strand RNA driver.

[Claim 10] The method according to claim 8, wherein said enzyme is single-strand-specific RNA endonuclease.

[Claim 11] The method according to claim 8, wherein said enzyme is either selected from the group consisting of RNase 1, RNaseA, RNase4, RNaseT1, RNaseT2, RNase2, and RNase3, or a mixture thereof.

[Claim 12] The method according to claim 8, wherein said enzyme is RNase 1.

[Claim 13] The method according to any of claims 1-11, wherein said cDNA tester is prepared by CAP-trapping the 5' end of RNA.

[Claim 14] The method according to any of claims 1-12, wherein the preparation of said cDNA tester comprises the following steps:

(1) synthesizing first strand cDNA by means of reverse transcriptase forming mRNA/cDNA hybrids;

(2) chemically binding a tag molecule to the diol structure of the 5' CAP($^{7Me}G_{ppp}N$) site of mRNA forming hybrids;.

(3) trapping long-strand, full-coding, and/or full-length cDNA hybrids; and

(4) removing single strand mRNA through digestion with an enzyme capable of cleaving single strand mRNA.

[Claim 15] The method according to claim 13, wherein said tag molecule is digoxigenin, biotin, avidin, or streptavidin.

[Claim 16] The method according to any of claims 1-14, wherein said normalization driver is cellular mRNA from the same library as what is to be normalized, cellular mRNA from the same tissue as what is to be normalized, or cellular mRNA from the same cDNA population as what is to be normalized.

[Claim 17] The method according to any of claims 1-14, wherein said normalization driver is single strand cDNA obtained from the same library, the same tissue, or the same cDNA population as what is to be normalized.

[Claim 18] The method according to any of claims 1-14, wherein said subtraction driver is cellular mRNA from a library, tissue, or cDNA population differing from what is to be subtracted.

[Claim 19] The method according to any of claims 1-14, wherein said subtraction driver is single strand cDNA from a library, tissue, or cDNA population differing from what is to be normalized.

[Claim 20] A method of preparing normalized and/or subtracted cDNAs which comprises:

(a) a step of preparing cDNA testers;

(b) a step of preparing normalization and/or subtraction RNA drivers;

(c) a step of conducting normalization and/or subtraction in two steps in any order, or conducting normalization/subtraction as a single step, by mixing the normalization/subtraction RNA driver with said cDNA tester;

(d) a step comprising addition of an enzyme capable of cleaving single strand sites on RNA drivers non-specifically bound to cDNA tester;

(e) a step of removing said single strand RNA driver cleaved in step d) from the tester and removing tester/driver hybrids; and

(f) a step of recovering the normalized and/or subtracted cDNA.

[Claim 21] The method according to claim 19, wherein said cDNA tester is single strand or double strand.

[Claim 22] The method according to claim 19, wherein said cDNA tester is sigle strand and said method further compriese a step g) of preparing a second strand of recovered cDNA and performing cloning.

[Claim 23] The method according to any of claims 19-21, wherein in step d), the enzyme is RNase 1.

[Claim 24] The method according to any of claims 2-22, wherein said tester/driver hybrid binds to a tag molecule.

[Claim 25] The method according to claim 23, wherein said tag molecule is avidin, streptavidin, biotin, digoxigenin, an antibody, or an antigen.

[Claim 26] The method according to any of claims 2-24, wherein said tester/driver hybrids are removed through the use of a matrix.

[Claim 27] The method according to claim 25, wherein said matrix is magnetic beads or agarose beads.

[Claim 28] The method according to claim 26, wherein said magnetic beads or agarose beads are covered by or bound to any tag molecule capable of binding to tag molecules bound to a tester/driver hybrid.

[Claim 29] The method according to claim 26, wherein said magnetic beads or agarose beads are covered by or bound to a tag molecule capable of binding to avidin, streptavidin, biotin, digoxigenin, an antibody, or an antigen bound to a driver/tester hybrid.

[Claim 30] The method according to claim 27 or 28, wherein the antibody covering said beads or the antibody binding said beads is an antiantigen antibody, antibiotin antibody, antiavidin antibody, antistreptavidin antibody, or antidigoxigenin antibody.

[Claim 31] The method according to any of claims 2-29, wherein said tester/driver hybrid is removed through the use of streptavidin/phenol.

[Claim 32] The method according to any of claims 2-22, wherein hydroxyapadite and nonlabeled RNA are employed to remove said tester/driver hybrid.

[Claim 33] cDNA obtainable by the method according to any of claims 1-31.

[Claim 34] The cDNA according to claim 32 that is single strand or double strand.

[Claim 35] A method of removing RNA nonspecifically bound to DNA by processing nonspecifically bound RNA/DNA hybrids with an enzyme capable of degrading single strand RNA.

[Claim 36] The method according to claim 34, wherein said enzyme is either selected from the group consisting of RNase 1, RNaseA, RNase4, RNaseT1, RNaseT2, RNase2, and RNase3, or a mixture thereof.

[Claim 37] The method according to claim 34, wherein said enzyme is RNase 1.

[Claim 38] The method according to any of claims 34-36, wherein said RNA/DNA hybrid is a product of normalization method.

[Claim 39] The method according to any of claims 34-36, wherein said RNA/DNA hybrid is a product of subtraction method.

[Claim 40] The method according to any of claims 34-36, wherein said RNA/DNA hybrid is the product of a method comprising the steps of normalization and subtraction in any order or of a method comprising a single normalization/subtraction step.

[Claim 41] A method of isolating single strand cDNA comprising the steps of treating a hybrid comprising RNA nonspecifically bound to said cDNA with an enzyme capable of degrading single strand RNA, removing said degraded single strand RNA, and recovering said cDNA.

[Claim 42] A method of preparing normalized and/or subtracted cDNA comprising the steps of adding an enzyme capable of degrading single strand RNA driver nonspecifically bound to cDNA tester, and removing said degraded single strand RNA driver.

[Claim 43] The method according to any of claims 34-41, wherein said DNA or cDNA is long-chain, full-coding, and/or full-length cDNA.

[Claim 44] The method according to any of claims 1-31 and 34-42 employed to prepare one, two, or more libraries.

[Claim 45] cDNA or a cDNA library obtainable by the method according to any of claims 1-31 and 34-42.

[Detailed Description of the Invention]

[0001]

[Field of the Invention]

The present invention relates to an improved method of preparing normalized and/or subtracted cDNAs or cDNA libraries.

The present invention also relates to a method for improvement of the normalization and subtraction steps by eliminating non-specifically bound hybrids.

[0002]

[Related art and Problems to be solved by the Invention]

Methods of preparing cDNA libraries have been disclosed and are well known in prior art. For example, they are described by Ederly I., et al., 1995, Mol Cell Biol, 15:3363-3371; Kato S., et al., 1994, Gene, 150:243-250; and K. Maruyama et al., 1995, Gene, 138:171-174.

[0003]

Among them, Carninci et al., 1996, Genomics 37:327-336; Carninci et al., 1997, DNA Research, 4:61-66; and Carninci and Hayashizaki, 1999, Methods Enzymol, 303: 1-44, describe efficient methods for the preparation of cDNAs. These methods, comprising a modified "tagged cap trapper" to select long-strand, full-coding and/or full-length cDNA libraries after tagging of the cap structure, allow the preparation of long-strand, full-coding and/or full-length cDNA libraries containing all of a particular coding sequence and its 3' and 5' untranslated regions (UTRs). Such libraries are particularly useful for large-scale sequencing projects in which the recovery of long-strand, full-coding and/or full-length (full-coding/length) clones is required from among truncated clones (EST sequences).

[0004]

However, the preparation of long-strand, full-coding/length cDNA libraries entails certain problems. The preparation of long-strand or full-coding/length cDNA is more efficient for short-strand mRNAs than for long-strand mRNAs (transcripts). In addition, since cloning and amplification is more difficult for long-strand cDNAs than for short-strand cDNAs, length bias is further occurred. Using truncated cDNAs to recover full-length cognates is impractical at the genomic-scale level; however, cDNAs in a standard library can be cloned in either their long-straned, full-coding/length or truncated form, thus favoring discovery of at least one EST for any gene, regardless of length.

[0005]

Another problem relates to the nature of cellular mRNA. mRNA can be classified into "superprevalent mRNA (or highly expressed mRNA) ", "intermediately expressed mRNA" and "rarely expressed mRNA" based on expression. In a typical cell, 5-10 species of superprevalent mRNA are comprised in an amount of at least 20

percent of total mRNA, 500 to 2,000 species of intermediately expressed mRNA are comprised in an amount of 40 to 60 percent of total mRNA, and 10,000 to 20,000 species of rarely expressed mRNA are comprised in an amount of 20 percent to less than 40 percent of total mRNA. This average distribution varies markedly between tissue sources, and the presence of numerous highly expressed genes further alters this distribution. Sequencing cDNA from standard cDNA libraries is ineffective to discover rarely expressed genes because intermediately and highly expressed cDNA ends up being excessively sequenced.

[0006]

As mentioned above, when superprelevant mRNAs and intermediately expressed mRNAs are identified, redundancy levels are expected to exceed 60 percent. Thus, the use of a hybridization normalization method has been proposed to solve this problem. The principle behind normalization is to decrease the frequency of the most highly expressed clones while increasing the frequency of less prevalent cDNAs. Several methods of normalization for the preparation of EST cDNAs are introduced by Soares et al., 1994, Proc. Natl. Acad. Sci. 91:9228-9232, who has disclosed a normalization method for preparing EST sequences. This method is based on the reassociation of amplified plasmid libraries. However, plasmid libraries subjected to normalization are not suitable for the preparation of long-strand, full-coding/length cDNAs. This is because there is a cloning bias associated with plasmid libraries where short-strand cDNAs are efficiently cloned with cloning efficiency decreasing with the length of the strand. In fact, in Soares et al., 1994, DNA is cloned into a plasmid and then be converted to single-strand DNA. If the ligation to plasmids is accomplished, it becomes difficult to recover long-strand cDNAs (that is, long strands of cDNA tend to be lost).

[0007]

Additionally, during library amplification prior to normalization, the ease with which cDNA clones are grown varies with plasmid length. Thus, long-strand, full-coding/length clones tend to be underrepresented following bulk amplification of

the library. In plasmid libraries, the recovery of long-strand, full-coding/length clones becomes even more difficult.

[0008]

Other literature, such as Tanaka et al., 1996, Genomics, 35:231-235, discloses methods for the preparation of EST sequences in which mRNA is first annealed on oligo-dT conjugated on a solid matrix. This method is not suitable for preparing normalized long-strand, full-coding/length cDNAs because of mRNA degradation before cDNA synthesis. Further, the hybridization rate of nucleic acids immobilized on a solid phase is slower than that in solution hybridization.

Libraries created with PCR- and solid matrix-based normalization technologies known in the art exhibit sequence redundancy similar to that of non-normalized cDNA libraries used in EST projects.

[0009]

An additional problem consists in that in the preparation of cDNA libraries or encyclopedias (for example, a mammal full-length cDNA encyclopedia) or in the preparation with the aim of collecting at least one long-strand, full-coding/length cDNA for each gene expressed irrespective of the tissue source, not only is it desirable to remove cDNAs that are redundant within the library, but also cDNAs that have already appeared in previous libraries, so as to accelerate the discovery of new long-strand, full-coding/length cDNAs.

[0010]

To solve this problem, hybridization subtraction methods have been proposed.

Sagerstrom et al., Annu. Rev. Biochem., 1997, 66:751-83, gives an overview of the subtraction methods known in the art. The basic idea of subtraction is that the nucleic acid of the expressed sequence that is to be isolated and remains after subtraction (the tracer or tester) is hybridized to complementary nucleic acid that is believed to lack sequences of interest (drivers) and in which the drivers are present in much higher concentration than thetesters. The tracer and driver nucleic acid populations are allowed to hybridize, with only sequences common to the two populations forming hybrids. After hybridization, driver-tracer hybrids and

unhybridized drivers are removed, and the remaining nucleic acids can be used to prepare a library rich in tracer-specific clones or to make probes that can be used to screen a library for tracer-specific clones.

[0011]

On the other hand, subtraction methods also entail the same problems described for normalization with PCR- and solid matrix-based technologies. They are suited to the preparation of EST sequences, but cannot be used to prepare long-strand, full- coding/length cDNAs.

[0012]

Bonaldo et al., 1996, Genome Reseach, 6:791-806, discloses a subtractive hybridization specifically applied to reducing the expression of populations of already sequenced clones from normalized libraries yet to be surveyed.

[0013]

However, this normalization and subtraction technique (Bonaldo et al., 1996) is useful for large-scale gene discovery in EST research, but has the drawbacks already indicated in prior art, and is not suited to long-strand and full- coding/length cDNA inserts.

[0014]

Actually, as stated above, during library amplification prior to the normalization and subtraction steps, the amplification of cDNA clones varies with plasmid length, with expression of long clones being decreased following bulk amplification of the library. That is, the expression of long-strand cDNA clones decreases relatively, rendering such cloning difficult.

[0015]

A further problem of the normalization and subtraction method disclosed by Bonaldo et al. is that both the normalization and subtraction steps require incubation and an incubation period causing the breakup of plasmids -- bigger plasmids (containing long-strand cDNAs) in particular. As a consequence of these two steps, the number of long-strand clones is very limited or null.

[0016]

This also confirms the unsuitability of this method to the preparation of normalized and subtracted long-strand, full-cloning/length cDNAs.

A still further problem relating to normalization and/or subtraction methods relates to the fact that non-specifically-bound tracer (tester)/driver hybrids are formed in these steps due to complementary binding of imperfect sequences. The removal of such hybrids would result in the elimination from the tester of targeted cDNAs erroneously considered to be abundant and/or to have already been sequenced in other libraries, but which in reality are not prevalent and have not been previously sequenced.

[0017]

Accordingly, the purpose of the present invention is to solve the several problems of prior art and to provide an efficient method for the preparation of normalized and/or subtracted long-strand and full-coding/length cDNA libraries.

[0018]

[Means for solving problems]

The present invention provides a procedure (methodology) capable not only of normalizing cDNA, but also of subtracting cDNAs that have already appeared in other libraries. Accordingly, aspects of the present invention provide an efficient method for the preparation of normalized and/or subtracted cDNAs, preferably, long-strand and/or full-coding/length cDNA or cDNA libraries. Based on this method, the problems of PCR- and solid matrix-based techniques are solved.

[0019]

Accordingly, one aspect of the present invention relates to a method of preparing normalized and/or subtracted cDNAs, characterized in that the normalized and/or substracted cDNA is reverse transcript of mRNA in the form of uncloned cDNA (hereinafter referred to as cDNA tester).

[0020]

Furthermore, another aspect of the present invention comprises the following steps:

I) preparing cDNA testers, preferably long-strand, full-coding/length cDNAs;

II) preparing polynucleotide drivers for normalization and/or subtraction;

III) normalizing and/or subtracting (one, two, or more steps), removing the tester/driver hybrids obtained by normalization and/or subtraction, and removing unhybridized polynucleotide drivers; and

IV) recovering the normalized and/or subtracted cDNA.

[0021]

In the method of the present invention, cDNA tester can be single strand or double strand. When cDNA tester is single strand, the method of the present invention may comprise the step V) of preparing a second strand of cDNA and performing cloning. In addition, when cDNA tester is double strand, the method of the present invention may comprise the step V) of cloning the double-strand cDNA that is recovered.

[0022]

According to another aspect, the present invention relates to a method of preparing cDNA, preferably long-strand, full-coding/length cDNA, wherein the normalization and subtraction drivers are mixed together and normalization and subtraction are performed in a single step (normalization/subtraction).

[0023]

According to a further aspect of the present invention, a method of improving normalization and/or subtraction by eliminating RNA (driver) bound non-specifically to cDNA (tester) by treating the non-specific binding RNA/DNA hybrid with an enzyme capable of cleaving single-strand sites in RNA drivers is provided. This enzyme can be a nuclease, in particular, a ribonuclease capable of cleaving single-strand RNA or a mixture thereof. Preferably RNase 1 can be employed.

[0024]

However, this treatment is not limited to the normalization and subtraction of hybrids in the method of preparing cDNA, but can be used to cleave sites in RNA that has become partly single-stranded as a result of nonspecific binding and thus selectively remove nonspecifically bound RNA/DNA hybrids in any species of nonspecifically-bound RNA/DNA hybrid.

[0025]

Accordingly, a method of preparing single-strand and/or double-strand cDNA by treating nonspecifically bound RNA/DNA hybrids with an enzyme capable of cleaving single-strand RNA (capable of cleaving sites in RNA that has become partly single-stranded), removing the cleaved RNA, and recovering the cDNA is provided.

[0026]

The normalized and/or subtracted cDNA prepared by any of the methods of the present invention may be single-strand or double-strand cDNA.

[0027]

[Modes for carrying out the Invention]

According to one aspect of the present invention, an efficient method for the preparation of normalized and/or subtracted cDNA or cDNA libraries, preferably long-strand and/or full-coding/length cDNA libraries, is provided.

[0028]

This method does not require the PCR cloning step and the binding of oligo-dT to solid matrix before the normalization and/or subtraction step(s) (one, two, or more steps). As a result, long-strand and/or full-coding/length cDNAs are recovered.

Accordingly, the method of the present invention may comprise only the normalization step, only the subtraction step, both the normalization and subtraction steps in any order, or normalization and subtraction performed simultaneously in a single step.

[0029]

These normalized and/or subtracted cDNAs are treated to synthesize a complementary second strand when they are single-strand, and finally cloned.

Accordingly, the method according to this aspect comprises the steps of:

I) preparing a cDNA tester, preferably a long-strand and/or full-coding/length cDNA tester;

II) preparing polynucleotide (driver) for normalization and/or subtraction;

III) performing the normalization and/or the subtraction step(s) (one, two, or more steps), and removing the tester/driver hybrids obtained and the non-hybridized polynucleotide drivers; and

IV) recovering normalized and/or subtracted cDNA (rare and/or new cDNAs).

[0030]

The method based on the present invention further comprises, when the cDNA tester is single-strand, a step V): preparing the second strand of cDNA and performing cloning.

[0031]

For the purposes of the present invention, the term "full-length cDNA" denotes 5' and 3' UTR sequences and T-primer oligonucleotides (that are complementary to mRNA comprising poly-A). "Full-coding cDNA" means a cDNA sequence comprising at least a start codon and a stop codon. And by "long-strand cDNA" is understood a cDNA sequence which is almost full-coding and/or full-length but lacks one or more bases at the 3' end (corresponding to the 5' end of mRNA) or at the 5' end if considering a cDNA strand complementary to cDNA that is complementary to mRNA (that is, cDNA having the same direction as the gene). This early stop (before reaching the 5' end) can be caused by the formation of a secondary structure in the mRNA at the Cap structure level, impeding synthesis of cDNA.

[0032]

The step I) can be performed by any method of cDNA preparation known in prior art, preferably any known method for the preparation of long-strand and/or full-coding and full-length cDNAs. Examples are Ederly et al.; Kato et al.; and Maruyama et al.(the oligo-capping method).

[0033]

In particular, the oligo-capping method is a method where phosphoric esters of incomplete cDNAs without 5' Caps are removed with alkaline phosphatase and then all cDNAs are treated with the tobacco mosaic virus (TAP) used as decapping enzyme so that only full-length cDNAs have phosphates.

[0034]

Preferably, a method employing the CAP-trapping technique described by Carninci et al., 1996, Genomics 37:327-336, and Carninci and Hayashizaki, 1999, Methods Enzymol, 303: 1-44, is used.

An example of this method is schematically illustrated in section A of Figure 1.

[0035]

mRNA is isolated from tissue and RNA-DNA hybrids are made by reverse transcriptase starting from primers such as oligo-dT or random or specific primer-adapters, using mRNA as template. A tag molecule is then chemically bound to the diol structure of the 5' CAP ($^{7Me}G_{ppp}N$) site of the mRNA forming the hybrid. Finally, RNA-DNA hybrids carrying a DNA corresponding to long-strand, full-coding/length mRNA are separated from hybrids carrying tag molecules by binding the tag molecules.

[0036]

Tag molecules that are bound to the Cap after formation of the RNA-DNA hybrids are particularly advantageous because the hybrid structure of RNA-DNA can escape chemical cleavage of mRNA during the aldehydration of the diol structure necessary for labelling mRNAs with tag molecules. As a result, the efficiency of full-coding/length cDNA synthesis increases.

[0037]

The binding of tag molecule to the 5' Cap site can be progressed by, for example, an oxidation ring-opening reaction of the 5' Cap site diol structure with an oxidizing agent such as sodium periodate to form a dialdehyde and subsequent reaction of the dialdehyde with a tag molecule having a hydrazine terminal.

[0038]

Examples of tag molecules having hydrazine terminals are biotin, avidin and streptavidin, as well as digoxigenin molecules having hydrazine terminals. A molecule showing reaction specificity, such as antigens and antibodies, can also be used as tag molecule. The label molecule employed as tag molecule is not specifically limited.

[0039]

Accordingly, the preparation of cDNA used to practice any of the methods based on the present invention comprises the steps of:

(1) synthesizing a first strand of cDNA by reverse transcriptase forming the hybrid mRNA/cDNA;

(2) chemically binding a tag molecule to the diol structure of the 5' CAP ($^{7Me}G_{ppp}N$) site of the mRNA forming the hybrid;

(3) capturing long-strand, full-coding, and full-length cDNA hybrids; and

(4) removing single-strand mRNA by digestion with an enzyme (preferably with RNase H) that is capable of cleaving single strand mRNA or by using an alkali (preferably NaOH).

[0040]

A more specific example of the aforementioned method (a more specific example for preparing cDNA) including steps from (1) synthesizing first cDNA strands to (7) synthesizing double-stranded full-coding/length cDNA (having for example biotin as tag molecule) is as follows:

(1) synthesis of first strand cDNA (synthesis of an RNA-DNA hybrid);

(2) biotinylation of an mRNA of the RNA-DNA hybrid;

(3) ribonuclease I (RNase I) digestion;

(4) capture of a full-coding/length cDNA hybrid (with avidin or streptavidin beads);

(5) removal of hybrid RNA (RNase H digestion);

(6) G tail addition by terminal deoxynucleotidyl transferase; and

(7) preparation of second strand (double stranded full-coding/length cDNA) primed with oligo C.

[0041]

For example, step (5) can be performed with an alkali (preferably NaOH). The single strand cDNA obtained by the method of step I) consists of various populations of cDNA, namely superprevalent (or highly expressed or class I), intermediate (or class II) and rare cDNAs (class III) as indicated in section B of Figure 1 (intermediate and superprevalent are indicated together in section B as abundant). Among some of these cDNAs, cDNAs that have been previously collected in other libraries and are indicated

in section B must be considered as previously collected cDNA. The cDNAs that were obtained are indicated in section B as "testers".

[0042]

In step II), "drivers" are prepared for normalization and/or subtraction.

Normalization drivers are RNA or DNA obtained from the same tissue as the population, and/or the same population, that one intends to eliminate.

Normalization drivers can be for example cellular mRNAs of the same library, that is, aliquots of the mRNAs initially used to prepare the cDNA library (starting material mRNA). Normalization drivers can also be cDNA obtained from the same library that one intends to normalize. In that case, single-strand cDNAs are prepared from the cDNA library by PCR, for example.

[0043]

Subtraction drivers can be RNA or DNA obtained from different tissue from that which one intends to substract, RNA or DNA obtained from the same or a different strain (system) from that which one intends to subtract, or the same tissue belonging to a different population.from the population that one intends to eliminate.

In vitro-transcribed RNA from a DNA library, preferably clones from different tissue or from the same tissue but belonging to a different population and prepared with the cap-trap technology, can be employed in the subtraction step. However, subtraction drivers prepared with any method known in the art, for example, as described by Sagerstrom et al., 1997, Annu. Rev. Biochem., 66:751-83, can be used for the purpose of the present invention.

[0044]

For example, subtraction drivers can be run-off transcripts from minilibraries containing expressed genes, rearrayed clones, and in some cases, previously sequenced (but not necessarily) cDNAs.

Subtraction run-off transcripts are obtained by RNA polymerase (for example, T7, T3 , SP6 or K11 RNA polymerase) from DNA templates carrying appropriate promoters, such as a DNA sequence flanked with promoters, plasmids, phages, and analogs thereof. In the case of plasmids, subtraction transcripts can be prepared by

amplifying the cDNA library or rearrayed library with a solid or liquid phase. Preferably, subtraction transcripts can be prepared by spotting colonies obtained from well plates (for example 384 well plates) onto LB+ ampicillin-agar plates, growing them at a temperature of from 30°C to 37°C, and on the following day, scraping the colonies for use in bulk plasmid preparation.

[0045]

DNA can also be used for subtraction drivers. In that case, single-strand DNA isolated from clones obtained from different tissues or from the same tissue but belonging to a different DNA population can be used.

Minilibraries are libraries comprising a portion of the clones of the starting tissue or of different tissues.

[0046]

A schematic example of the preparation of drivers for normalization and/or for subtraction is shown in section C of Figure 1. However, methods based on the present invention comprise only the normalization step, only the subtraction step, the normalization and subtraction steps in any order, or normalization/subtraction in a single step. These drivers can be ones bound with a tag molecule. The tag molecule can be bound to any molecule that is able to bind or a driver as a tag and also be able to bind to a matrix permitting elimination of the driver. Preferred tag molecules are biotin, avidin, strepavidin, digoxigenin, or any antibody thereto, preferably an antibiotin, antiavidin, antistrepavidin or or antidigoxigenin antibody, or any antiantigen antibody thereof. However, the tag molecule is not limited to these substances.

[0047]

Steps III) and IV) can be executed in different order depending on whether the normalization and subtraction steps are carried out consecutively or in a single step.

According to a first approach, normalization step (III) is performed by mixing with normalization drivers, followed by elimination of the hybrids and recovery of the normalized single-strand cDNAs (step IV). Next, these single-strand cDNAs are further mixed with subtractive drivers (step III), the hybrids are eliminated and (normalized and) subtracted single strand cDNAs are recovered (step IV). The

single-strand cDNAs that are recovered also comprise rare and new cDNAs as exemplified in section D of Fig.1. The normalization and subtraction steps mentioned above can also be performed in inverted order.

[0048]

According to another approach, the normalization drivers and the subtractive drivers prepared in step II) are mixed together, with a single normalization/subtraction step being performed in step III).

The implementation of normalization/substraction in a single step affords the advantage of performing only one incubation step. If increasing the number of incubation steps, the number of long-strand and/or full-coding/length cDNAs tends to decrease. Thus, performing only a single incubation step is advantageous.

[0049]

However, the normalization and/or subtraction step and the single normalization/subtraction step can be repeated, if necessary, before final recovery of the rare new cDNAs and synthesis of second strand cDNA.

[0050]

The preparation of normalization and/or subtractive drivers, the hybridization step(s) (one or more), and the removal of "unnecessary cDNAs", i.e. hybrids produced in the normalization and/or subtraction steps and single drivers (drivers not forming hybrids) can be accomplished by any technique known to the art, for example, those described in Bonaldo et al., 1996, and in Sagerstrom et al. 1997, Annu. Rev. Biochem., 66:751-783 (from page 765; also Table 1).

[0051]

As a specific example, the hybridization technique relating to photoactivated biotin, strepavidin binding and organic extraction described by Barr F.G. and Beverly S. Emanuel, 1990, Analytical Biochemistry, 186: 369-373 or in Hazel L. Sive and Tom St John, 1988, Nucleic Acids Research, Vol. 16, number 22, from page 10,937, can be employed.

However, techniques known in the art such as are described by Sagerstrom et al., 1997, may be employed.

[0052]

Following normalization and/or subtraction the tester/driver hybrids are removed by any technique known in the art, such as that described by Sagerstrom et al., 1997, from page 765. For example, a matrix such as beads, preferably magnetic beads or agarose beads can be employed. The beads are preferably covered with a tag molecule, as set for above, or bind to tag molecules. Beads covered with streptavidin (generally referred to as "streptavidin beads") are preferred, with magnetic porous glass (MPG) streptavidin beads (CPG Inc.) being even more preferred. Beads covered with or bonded to avidin, biotin, digoxigenin, an antibody, or an antigen can also be used. The antibody covering or bound to the beads (one, two, or more types of beads) can be an antibody generally able to recognize tag molecules, preferably an antibody which recognizes antibody bound to the drivers, or an antibiotin antibody, antiavidin antibody, antistreptavidin antibody, or antidigoxigenin antibody which recognizes biotin, avidin, streptavidin or digoxigenin bound to the drivers.

[0053]

An example of magnetic beads bound to or covered with biotin as a tag molecule forming a tester/driver hybrid aggregate is shown in section F of Fig.1.

Streptavidin or avidin/phenol may be employed instead of magnetic beads to remove the hybrid (Sive H.L. and St.John T., 1988, Nucleic Acids Res., 16:10937; and Schneider C. et al., 1988, Cell, 54: 787-93).

It is also possible to employ hydroxyapatite (HAP) and unlabeled RNA to remove tester/driver hybrids. An example is described by Sagerstrom et al., page 765 and Table 1.

[0054]

The removal of tester/driver hybrids by the subtraction method based on the present invention permits almost 100 percent removal as seen from the electrophoresis of Figure 6 and from Example 3.

The cDNA of the removed tester/driver hybrids can be used for the preparation of cDNA minilibraries to be used for further subtractions steps as shown in section F of Fig.1.

[0055]

The normalized and subtracted cDNAs (rare and new cDNAs) that are recovered in step IV) are then treated to synthesize second cDNA strands, subjected to restriction digestion, ligated, and cloned into vectors, as schematically indicated in section E of Fig.1.

The advantage of the method based on the present invention is that it maintains a high proportion of long-strabd, full-coding/length cDNAs in the subtracted/normalized library. Further, the present method can increase the discovery of new genes relative to the results obtained by using standard, full-length cDNA libraries prepared based on prior art.

That is, in the method of the present invention, normalized and/or substracted cDNA (tester cDNA) is a reverse transcript of mRNA in the form of uncloned cDNA. This cDNA can be a single-strand or double-strand cDNA. Thus, the prior art problem of cloning bias against size of cDNAs in plasmid libraries and the problem in libraries generated by normalization techniques based on PCR and solid matrixes can be avoided, and the advantage of increased discovery of new genes is afforded.

[0056]

As stated in the related art section, a further problem relating to normalization and/or subtraction methods is that non-specific tester/driver hybrids form during these steps due to complementarity binding of imperfect sequences. For example, this is caused by cross-reactivity between similar but unidentical sequences in testers and drivers. The removal of such hybrids eliminates from the tester cDNAs those cDNAs considered to be erroneously abundant and/or to have been already sequenced in other libraries, as well as other desirable sequences. This constitutes a major drawback to normalized/subtracted libraries.

[0057]

This problem is schematically depicted in Figure 2. On the left side of Fig.2, normalization and/or subtractive drivers (mRNA, upper strands) are non-specifically bound (there is a portion of the mRNA that is not bound to the cDNA) to cDNAs (lower strands) that are new and/or rare but erroneously believed to be abundant and/or

to have been previously collected. cDNA testers that are bound to drivers, albeit nonspecifically, are removed during normalization and/or subtraction as indicated in Fig. 1. Thus, when the cDNA testers are new and/or rare cDNAs, they are lost.

In the method of the present invention, in such nonspecific binding, such as is shown on the right side of Fig. 2, drivers that are nonspecifically bound to cDNA testers are eliminated by degrading RNA with RNase 1 (also referred to as RNase I) or some other enzyme described further below and the new and/or rare cDNA is recovered.

[0058]

As shown in Fig. 2, one aspect of the present invention provides a method in which nonspecifically binding RNA/DNA hybrids are processed (digested) by an enzyme cleaving single-strand RNA (an enzyme having the ability to cleave single-strand sites in RNA drivers nonspecifically bound to cDNA testers) to cleave single-strand sites of RNA (drivers) nonspecifically bound to cDNA (testers). Next, hybrids with RNA that has been cleaved from the cDNA testers are eliminated (denatured) to remove the cleaved RNA from the system, leaving the cDNA testers and improving the efficiency of normalization and subtraction (that is, reducing unintended exclusion from the rare cDNA system).

[0059]

There is no limitation to the method of removing RNA fragments from the nonspecifically bound RNA/DNA hybrids that have been treated with enzymes cleaving single-strand RNA. For example, as shown in Fig. 2, the hybrids may be processed at a suitable temperature to eliminate, that is, denature, hybrids with RNA that has been cleaved from cDNA tester, and the RNA freed from the cDNA tester may be removed using tags bound to the RNA, for example. The elimination of hybrids with RNA that has been cleaved from cDNA tester does not affect specifically bound RNA/DNA hybrids. The conditions for selectively denaturing hybrids that have been cleaved and the strand length of the hybrid portion that has been shortened are suitably selected. Denaturing conditions depend not just on temperature, but also on the pH of the system (the aqueous solution containing the hybrids), salt concentrations, and the

like. Denaturing is conducted for example by processing at a temperature of 25-95°C, preferably 37-70°C, and still more preferably at a temperature of 65°C. The use of the above-stated temperature denatures hybrids of partially cleaved RNA, the RNA previously constituting the hybrids dissociates from the cDNA, the dissociated RNA is bound to beads using tags bound to the RNA, and the RNA fragments bound to the beads can be removed with a magnet by the usual methods. Specifically bound RNA/DNA hybrids are maintained under the above-stated denaturing conditions, and tags bound to the RNA in the same manner as set forth above may be used for binding to beads and removal. That is, conventional normalization and/or subtraction are employed.

[0060]

A single-strand-specific RNA endonuclease (ribonuclease) can be employed as the above-described enzme cleaving single-strand RNA. Examples suitable for use are RNaseA specific to pyrimidine (U and C), RNase 4 specific to U, RNase T1 specific to G, RNase 2 or RNase 3 specific to U, and RNase 1 (also referred to as RNase I) able to degrade any ribonucleoside (Hyone-Myong Eun, Chapter for "Nucleases"; Sorrentino Salvatore and Libonati Massimo, 1997, FEBS Letters, 404:1-5). Alternatively, RNase T2 having little base specificity can be used as the enzyme cleaving single-strand RNA (BioTechniques, 232, Vol.12, No.2, 1992).

[0061]

RNase 1 is employed with preference as the enzyme cleaving single-strand RNA. A mixture of the above-listed ribonucleases may also be employed. Hybrids may be subjected to the action of single-strand-specific RNA endonucleases by the usual methods. For example, 0.01-1 unit of a single-strand-specific RNA endonuclease may be acted per 1 μ g of driver.

The step of degrading mRNA drivers nonspecifically bound to cDNA testers may be conducted in the normalization/subtraction step based on the present invention, following the normalization and/or substraction step, or after a single normalization/substraction step.

[0062]

Accordingly, one aspect of the the present invention is a method specifically comprising:

(a) a step of preparing cDNA testers;

(b) a step of preparing normalization and/or subtraction RNA drivers;

(c) a step of performing normalization and/or subtraction in two steps in any order, or performing normalization/subtraction in a single step by mixing normalization/ subtraction RNA drivers with cDNA testers mentioned above;

(d) a step comprising addition of an enzyme capable of cleaving single strand sites on RNA drivers nonspecifically bound to cDNA testers;

(e) a step of removing the cleaved single-strand RNA drivers of step d) from the testers and removing tester/driver hybrids;

(f) a step of recovering normalized and/or subtracted cDNAs; and

(g) a step of preparing second strand cDNAs and then cloning the recovered cDNAs when cDNA testers are single strand.

[0063]

The processing methods for removing single-strand RNA drivers can be applied beyond the normalization and substraction of hybrids in methods of preparing cDNA. For example, they can be used to remove partially single-strand RNA in all types of nonspecific RNA/DNA hybrids.

[0064]

That is, the present invention comprises a method of treating nonspecifically bound RNA/DNA hybrids with an enzyme capable of degrading single-strand RNA to remove RNA that is nonspecifically bound to DNA. In this method, nonspecifically bound RNA/DNA hybrids are treated with an enzyme capable of degrading single-strand RNA to degrade RNA nonspecifically bound to DNA and remove it from mixtures of DNA and/or RNA/DNA hybrids specifically bound to DNA. This method may be employed with the object of recovering DNA nonspecifically bound to RNA or with the object of recovering just DNA hybrids specifically bound to RNA.

In the present method, as set forth above, the enzyme capable of degrading single-strand RNA may either be selected from the group consisting of RNase 1, RNaseA, RNase4, RNaseT1, RNaseT2, RNase2, and RNase 3, or may be a mixture thereof, with RNase 1 being preferred.

[0065]

The RNA/DNA hybrids including the above-described nonspecifically bound RNA/DNA hybrids may be a product of normalization, a product of subtraction, a product of a method comprising normalization and subtraction steps conducted in any order or a product of a method comprising a single step of normalization/subtraction. That is, nonspecifically bound RNA/DNA hybrids may be removed from RNA/DNA hybrids obtained by conventional normalization and/or subtraction methods and the recovery rate of long-strand or rare cDNA can be improved. Accordingly, the cDNA forming the above-described nonspecifically bound RNA/DNA hybrids can be long-strand, full-coding and/or full-length cDNA.

[0066]

The present invention further includes a method of isolating single-strand cDNA in which the above-described hybrids comprising RNA nonspecifically bound to cDNA is treated with an enzyme capable of degrading single-strand RNA, the degraded single-strand RNA is removed, and the DNA is recovered. In this method, the (degraded) single-strand RNA that is produced by treatment with an enzyme capable of degrading single-strand RNA is removed from the system comprising the hybrids, resulting in the recovery of single-strand cDNA. Based on this method, cDNA nonspecifically bound to certain RNA may be selectively recovered.

In this method, in the same manner as above, the enzyme capable of degrading single-strand RNA can be either selected from among the group consisting of RNase 1, RNaseA, RNase4, RNaseT1, RNaseT2, RNase2, and RNase3, or can be a mixture thereof, with RNase 1 being preferred. Further, the cDNA may be long-strand, full-coding, and/or full-length cDNA.

[0067]

The present invention further includes a method of preparing normalized and/or subtracted cDNA in which an enzyme capable of degrading single-strand RNA driver nonspecifically bound to cDNA tester is added and the degraded single-strand RNA driver is removed. In this method, cDNA tester and hybrids comprising single-strand RNA driver nonspecifically bound to the cDNA tester are subjected to the action of an enzyme capable of degrading single-strand RNA driver to degrade, and following degradation, the degraded single-strand RNA driver is removed from the system comprising the hybrids.

In this method, in the same manner as above, the enzyme capable of degrading single-strand RNA can be either selected from among the group consisting of RNase 1, RNaseA, RNase4, RNaseT1, RNaseT2, RNase2, and RNase3, or can be a mixture thereof, with RNase 1 being preferred. Further, the cDNA may be long-strand, full-coding, and/or full-length cDNA.

[0068]

The method of the present invention may be employed to prepare one, two, or more cDNA libraries. The present invention also covers the cDNA and cDNA libraries obtained by the methods of the present invention.

[0069]

Finally, the methods according to the aspects the present invention permit the following:

(i) high-efficiency removal of mRNA drivers;

(ii) no cDNA size reduction that would affect the frequency of long-strand, full-coding/length cDNAs;

(iii) suitability to both normalization and subtraction;

(iv) low cross-reactivity between similar but unidentical sequences; and

(v) high-level reproducibility in terms of both the size of the drivers prepared and the number of libraries as well as ease of handling.

[0070]

[Examples]

The methods and embodiments of the present invention will be further described with reference to the following examples.

Example 1

Preparation of RNA

Slices of brain tissue (0.5-1g) were homogenized in 10 mL of solution D (Chomczynski , P. and Sacchi, N., 1987, Annal. Biochem., 162:156-159) and extracted with 1 mL of 2M sodium acetate (pH 4.0) and the same amount of a mixture of phenol/chloroform (volume ratio 5:1). After extraction, the same volume of isopropanol was added to the aqueous phase to precipitate RNA. This sample was incubated on ice for an hour and centrifuged at 4000 rpm for 15 minutes with cooling to collect the precipitates. The precipitates were washed with 70 percent ethanol and dissolved in 8 mL of water. A 2 mL quantity of 5M NaCl and 16 mL of an aqueous solution (pH 7.0) comprising 1 percent CTAB (cetyltrimethylammonium bromide), 4M urea, and 50 mM Tris were added to precipitate RNA and the polysaccharides were removed (CTAB precipitate). After centrifugation at 4,000 rpm for 15 minutes at room temperature, the RNA that had been obtained was dissolved in 4 mL of 7M guanidine-Cl. Double the quantity of ethanol was then added to the solution and the mixture was incubated for an hour on ice and centrifuged at 4,000 rpm for 15 minutes. The resulting precipitates were washed with 70 percent ethanol and collected. The precipitates were again dissolved in water and the purity of the RNA was determined by measuring the OD ratio 260/280 (>1.8) and 230/260 (<0.45).

[0071]

Synthesis of cDNA

From 5 to 10 μg of this RNA, 5 μg of first-strand primer containing the *Bam*HI and *Sst*I restriction sites (5'-$(GA)_5$AGGATCCAAGAGCTC$(T)_{16}$VN-3') (SEQ ID NO:1), and 11.2 μL of 80 percent glycerol were combined to a total volume of 24 μL. The RNA-primer mixture was denatured at 65°C for 10 min. In parallel, 18.2 μL of 5X first-strand synthesis buffer, 9.1 μL of 0.1 M DTT, 6.0 μL (each) of 10 mM

dTTP, dGTP, dATP, and 5-methyl-dCTP (instead of dCTP), 29.6 μL of saturated trehalose (approximately 80 percent, low metal content; Fluka Biochemika), and 10.0 μL of Superscript II reverse transcriptase (200 U/μL) were combined to a final volume of 76 μL. A 1.0 μL quantity of [α-^{32}P]dGTP was placed in a third tube. The mRNA, glycerol, and primers were mixed on ice with the solution containing the aforementioned Superscript, and an aliquot (20 percent) was quickly added to the tube containing the [α-^{32}P]dGTP. First-strand cDNA synthesis was performed in a thermocycler with a heated lid (e.g., MJ Research) according to the following program: step 1, 45°C for 2 min; step 2, gradient annealing: cooling to 35°C over 1 min; step 3, complete annealing: 35°C for 2 min; step 4, 50°C for 5 min; step 5, increase to 60°C at 0.1°C per second; step 6, 55°C for 2 min; step 7, 60°C for 2 min; step 8, return to step 6 and repeat for 10 additional cycles. Incorporation of radioactivity permits estimation of the yield of cDNA (Carninci and Hayashizaki, 1999). The cDNA obtained was treated with proteinase K, extracted with phenol/chloroform and chloroform, and ethanol-precipitated using ammonium acetate as the salt (Carninci and Hayashizaki, 1999).

[0072]

Biotinylation of mRNA

Before biotinylation, the diol group of the cap and 3'-end of the mRNA was oxidized in a final volume of 50 μL of suspended mRNA/first-strand cDNA hybrid, 66 mM sodium acetate (pH 4.5), and 5 mM $NaIO_4$. Samples were incubated on ice in the dark for 45 min. mRNA/cDNA hybrids were then precipitated by adding 0.5 μL of 10 percent SDS, 11 μL of 5M NaCl, and 61 μL of isopropanol. After incubation in the dark on ice for 45 min or at -20°C or -80°C for 30 min, the samples were centrifuged for 10 min at 15,000 rpm. Finally mRNA/cDNA hybrids were rinsed twice with 70 percent ethanol and resuspended in 50 μL of water. The cap was then

biotinylated in a final volume of 210 μL by adding 5 μL of 1M sodium acetate (pH 6.1), 5 μL of 10 percent SDS, and 150 μL of 10 mM biotin hydrazide long-arm (Vector Biosystem).

[0073]

Following overnight (10 to 16 hours) incubation at room temperature (22 to 26°C), the mRNA/cDNA hybrids were precipitated by adding 75 μL of 1M sodium acetate (pH 6.1), 5 μL of 5M NaCl, and 750 μL of absolute ethanol and incubated on ice for 1 hour or at -20 to -80°C for 30 min. The mRNA/cDNA hybrids were pelleted by centrifugation at 15,000 rpm for 10 min and the resulting pellet was washed once with 70 percent ethanol and once with 80 percent ethanol. The mRNA/cDNA hybrids were resuspended in 70 μL of 0.1X TE (1 mM Tris [pH 7.5], 0.1 mM EDTA).

[0074]

Capture and Release of Full-Length cDNA

A 500 μL quantity of MPG-streptavidin beads and 100 μg of DNA-free tRNA were combined and the mixture was incubated on ice for 30 min with occasional stirring. The beads were separated using a magnetic stand for 3 minutes and the supernatant was removed. The beads were then washed three times with 500 μL of washing/binding solution (2 M NaCl, 50 mM EDTA, pH 8.0).

[0075]

At the same time, 1 unit of RNase 1 (Promega) was added per 1μg of starting mRNA to the mRNA/cDNA hybrid sample in buffer supplied by the manufacturer (final volume 200 μL) and the sample was incubated at 37°C for 15 min. To stop the reaction, the sample was placed on ice and 100 μg of tRNA and 100 μL of 5M NaCl were added. To capture full-coding/length mRNA/cDNA hybrids, the biotinylated, RNase 1-treated mRNA/cDNA and the washed beads were combined and resuspended in 400 μL of the washing/binding solution. After mixing, the tube was

gently rotated for 30 min at room temperature. The full-coding/length cDNA remained on the beads, and the shortened cDNAs did not. The beads were separated from the supernatant on a magnetic stirrer. The beads were gently washed to remove nonspecifically adsorbed cDNA: two washes with washing/binding solution; one with 0.4 percent SDS and 50 μ g/mL tRNA; one with 10 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 40 μ g/mL tRNA, 10 mM NaCl, and 20 percent glycerol; and one with 50 μ g/mL tRNA in water.

The cDNA was released from the beads by adding 50 μ L of 50 mM NaOH and 5 mM EDTA and incubating for 10 min at room temperature with occasional mixing. The beads then were removed magnetically, and the extracted cDNA was transferred on ice to a tube containing 50 μ L of 1M Tris-HCl, pH 7.5. The dissolution cycle was repeated once or twice with 50 µL aliquots of 50 mM NaOH and 5 mM EDTA until most of the cDNA (80 to 90 percent, as measured by monitoring the radioactivity with a hand-held monitor) had been recovered from the beads.

[0076]

To remove traces of RNA that could later interfere with the biotinylated RNA, 100 μ L of 1 M Tris-HCl, pH 7.0, and 1 μ L of RNase I (10 U/μ L) were quickly added to the recovered cDNA on ice; the sample then was incubated at 37°C for 10 min. The cDNA was treated with proteinase K, phenol/chloroform-extracted, and back-extracted. Two to three μ g of glycogen were then added and the sample precipitated from ethanol in a siliconized tube. Alternatively the sample was concentrated by one round of ultrafiltration with a Microcon 100 (Millipore) for 40-60 min at 2,000 rpm. When precipitated from ethanol, the cDNA could be redissolved in 20 μ L of 0.1X TE.

[0077]

In this experiment, RNase H digestion was not conducted. However, hydrolysis was conducted with NaOH, which is capable of simultaneously hydrolyzing and denaturing double strands.

[0078]

CL-4B Spin-Column Filtration of cDNA

The cDNA samples were treated by CL-4B chromatography (Carninci and Hayashizaki, 1999) or on an S-400 spin column (Amersham-Pharmacia) essentially as described by the manufacturer.

[0079].

Oligo-dG Tailing of First-Strand cDNA

The cDNA sample, 5 μ L of 10X TdT buffer (2 M potassium cacodylate [pH 7.2], 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol), 5 μ L of 50 μM dGTP, 5 μ L of 10 mM $CoCl_2$, and 40 U terminal deoxynucleotidyl transferase were admixed to a final volume of 50 μ L. Samples were incubated at 37°C for 30 min. At the end, the reaction was stopped with EDTA 20 mM. The cDNA was treated with proteinase K, extracted with phenol chloroform, and precipitated in ethanol. The sample was finally redissolved in TE (10 mM Tris pH 7.5-8.0, EDTA 1 mM). After the tail length had been checked as described (Carninc and Hayashizaki, 1999), the cDNA was employed in second-strand synthesis for use in verifying libraries (see below) or for use in normalization and/or subtraction.

[0080]

Normalization Drivers

mRNA drivers comprising aliquots of starting mRNA are called "normalizing or normalization drivers." To calculate the concentration of the normalizing driver, the ribosomal/structural RNA contamination in the starting mRNA was approximated by assuming that the incorporation rate of the first-strand synthesis reflected the actual mRNA concentration, thus assuming 100 percent efficiency of priming and elongation. Assuming that the proportion of mRNA converted to first-strand cDNA corresponded to the actual mRNA concentration, less-than-full-length cDNAs were excluded from

consideration. Although not all of the mRNA is normally primed, a slight excess of normalization driver seldom has as dramatic an effect as a paucity of driver. Therefore, it was assumed that the amount of mRNA in the sample was the same as the quantity of first-strand cDNA produced.

[0081]

Subtraction Drivers

The subtractive drivers comprise bulk run-off transcripts prepared from cloned minilibraries and rearrayed libraries prepared from the nonredundant RIKEN cDNA encyclopedia using T7 and T3 RNA polymerases.

[0082]

The minilibraries contained approximately 1,000 to 2,000 clones of cDNA in the sample that were derived from previous normalization experiments conducted by the same methods as the experiment described in the present embodiment. Employing the standard protocol, minilibraries were prepared from the aliquot (highly expressed cDNA fraction) that was the by-product of normalization experiments. Following normalization, the highly expressed cDNA fraction was removed from the beads with 50 mM NaOH/5 mM EDTA. Following neutralization, second-strand cDNA was prepared. Cloning was accomplished in a manner analogous to what has been previously described (Carninci and Hayashizaki, 1999). Plasmid was then bulk-excised, and 1,000-2,000 clones per minilibrary were amplified on agarose/ampicillin. To prepare drivers, 20,000 to 50,000 colonies were plated (plate size 150 mm diameter) on SOB-agarose/ampicillin (Sambrook et al. 1989, "Molecular Cloning: A laboratory Manual" Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) and the plates were incubated overnight at 37°C. Bacterial cells were scraped from the plates in the presence of resuspension solution (Wizard DNA extraction kit; Promega) after which the manufacturer's protocol was followed.

[0083]

Preparation of Nonredundant cDNA Library Driver

Single clones from full-length cDNAs obtained in previous experiments conducted in the same manner as described in the present embodiment were rearrayed

for subtraction. Rearrayed cDNAs were coated from 384-well plates onto SOD agarose/ampicillin plates. Plasmid extraction, DNA cleavage, and RNA preparation were conducted in the same manner as for the minilibraries.

[0084]

When the library had been cloned at the *Sst*I site, the extracted plasmid was treated at multiple cloning sites on the 3'-end with *Sst*I. (In the case of mRNA extracted from the liver and lungs, the minilibrary was cloned with *Xho*I at the 3'-end site, and treated with *Pvu*I, instead.) RNA was prepared using either T3 or T7 RNA polymerase (Life Technologies), depending on the map of the construct used to prepare the driver, to prepare sense run-off RNAs. T3 polymerase was employed for *Pvu*I-cleaved minilibraries and T7 polymerase for *Sst*I-cleaved minilibraries. RNA was prepared using RNA polymerases (Life Technologies) in accordance with the manufacturer's instructions. Extensive digestion with 1 to 2 μL of DNaseI (RQ1, RNase-free, Promega) was performed for 30 min. Proteinase K digestion was then conducted, followed by extraction with phenol/chloroform and chloroform, and cDNA was precipitated.

[0085]

Biotin Labeling of Normalizing/Subtracting RNA Drivers

To further clean up RNA drivers before labelling, the RNeasy kit (QIAGEN) was employed in accordance with the manufacturer's instructions. Subsequently, the Mirus nucleic acid biotinylation kit (Panvera) was employed in essentially the manner described by the manufacturer. A 10 μg quantity of the RNA mix was labeled by combination with 10 μL of Label IT reagent and 10 μL of labeling buffer A to a final volume of 100 μL following the kit protocol instructions. The reaction was conducted by incubation at 37°C for 1 hour, after which the biotinylated RNA was precipitated by adding 1/20-volume of 5M NaCl and double volume of 99 percent ethanol. After standard ethanol precipitation, the pellet was washed once with 80 percent ethanol, resuspended in 20 μL of "1X Mirus labelling buffer A", and stored

at -80°C until use (alternatively, mRNA can be labelled using the psoralen-biotinylation kit (Ambion) in accordance with the manufacturer's instructions).

[0086]

Normalization/Subtraction

The RNA drivers and cDNA were deproteinated using proteinase K followed by phenol/chloroform extraction, chloroform extraction, and ethanol precipitation. Oligo-dG-tailed cDNA was used as a substrate, which was mixed with the RNA drivers and blocking oligonucleotides (biotin-dG_5 to $-dG_{30}$, here biotin-dG_{16} was used) to hybridize to the C-stretch present in the subtracting driver and with oligo-dT primer to block the polyA sequences. However, any oligonucleotide able to block common sequences between drivers and cDNAs can be used.

[0087]

Hybridization was typically carried out at RoT values of 1 to 500 (RoT is defined in examples by Sagerstrom et al., 1997) in a buffer containing 80 percent formamide (from a deionized stock), 250 mM NaCl, 25 mM HEPES (pH 7.5), and 5 mM EDTA. Various comprexities are obtained, 2.5 for rice, 1.0 for Drosophila, and 5 to 10, preferably 5 for mouse. Hybridization was carried out at 42°C in a dry oven; even volumes as small as 5 μ L did not require mineral-oil overlays. After hybridization, the sample was precipitated by adding 2.5 volumes of absolute ethanol and incubated for 30 minutes on ice. The sample was then centrifuged for 10 min at 15,000 rpm and washed once with 70 percent ethanol; the cDNA (both single-strand cDNA and mRNA/single-strand cDNA hybrids) was carefully resuspended in 10 μ L of water on ice.

[0088]

Treatment with RNase 1

As necessary, the tester/driver hybrids obtained in the above step can be treated with RNase 1 to remove the mRNA normalization and subtraction drivers bound nonspecifically to the tester cDNA.

After removing the supernatant from the sample, precipitated after hybridization as described above, the pellet was resuspended with 45 μ L of double-distilled water or TE 0.1 X (1 mM Tris, 0.1 mM EDTA, pH 7.5) on ice (to minimize nonspecific repeat annealing). The pellet was completely redissolved before proceeding to the next step.

[0089]

A 5 μ L quantity of 10 x RNase 1 Buffer (Promega) and 0.5 unit of RNase 1 were then added per 10 μ g of driver RNA.

The mixture was incubated at 37°C for 10 min, heated at 65°C for 10 min, and placed on ice. (As necessary, the samples can be treated with proteinase K, phenol/chloroform, and chloroform, and precipitated with ethanol before proceeding to the next step).

[0090]

Removal of Hybrid

The next step can be applied to the normalized/subtracted mixture whether or not it has been treated with RNase 1 as indicated in the above step.

In parallel, 50 μ L of MPG-strepavidin magnetic beads (CPG Inc.) were prepared for each 1 μ g of biotinylated driver RNA; a 5 μ L quantity of beads was found to be capable of binding more than 400 ng of biotinylated driver. To each 50 μ l of beads was added 10 μ g of tRNA as a blocking reagent and the beads were incubated at room temperature for 10 to 20 min or on ice for 30 to 60 min with occasional shaking. A magnetic stand was employed to remove the beads, and the beads were washed three times with a large excess of 1 M NaCl and 10 mM EDTA and resuspended in a volume of 1M NaCl and 10 mM EDTA equivalent to the original volume of the bead suspension.

[0091]

The blocked beads were mixed with the redissolved tester/driver mixture and the entire sample was incubated at room temperature for 15 min with occasional gentle mixing. After removing the beads using a magnetic stand for 3 min, the supernatant, which contained the single-strand normalized/subtracted cDNA, was recovered. The beads were washed once with an excess volume of binding buffer (1M NaCl, 10 mM EDTA) to recover any remaining ssDNA. The radioactivity of the labeled samples was measured before and after the procedure in order to estimate the yield of normalization/subtraction.

[0092]

Microcon 100 ultrafiltration was employed as described by the manufacturer (Millipore) to concentrate the cDNA solution to approximately 50 μ L. Subsequently, the cDNA was pelleted by the standard isopropanol procedure. The pellet was resuspended in 44 μ l of 0.1 x TE, to which 5 μ L of RNase 1 buffer and 1 U of RNase 1 were added to make a volume of 50 μ L. The samples were then incubated for 20 min at 37°C, after which 400 μ L of 0.2 percent SDS was added to inactivate the RNase 1. The residue of degraded RNAs, blocking oligonucleotide, SDS, and buffer were removed by ultrafiltration with a Microcon 100 filter at 2,000 rpm and 25°C until the volumes were reduced to less than 20 μ L. The samples were desalted by adding 400 μ l of 0.1X TE then centrifuging as above for a total of three washes. The cDNA was recovered by inverting the filter in a new tube and centrifuging at 9,000 rpm for 1 min.

[0093]

Synthesis of Second-Strand cDNA

The second-strand synthesis and cloning steps were identical for normalized/subtracted cDNA, the standard control libraries, and the minilibraries. In the same manner as for the first-strand cDNA primer, *Xho*I containing primer, 5'-$(GA)_7$TTCTCGAGTTAATTAAATTAATC$_{13}$-3' (SEQ ID NO:2) containing *Xho*I, was prepared and purified by standard techniques.

[0094]

To prepare the second-strand reaction, oligo-dG-tailed cDNA was mixed with 6 μ L of 100 ng/μ L second-strand primer adapter, 6 μ L of EX-Taq second-strand buffer (Takara), and 6 μ L of 2.5 mM (each) dNTPs. The reagents were combined with enzyme at 50°C (usually from 45°C to about 80°C) to ensure high specificity of priming (called "hot start"). Priming was then conducted by adding 3 μ L of 5 U/μ L ExTaq polymerase (Takara) at 65°C in a thermocycler. After mixing, the annealing temperature was set by a negative ramp to 45°C for the *Xho*I primer, and 35°C for the *Sst*I primer. After 10 minutes at the annealing temperature, the second-strand cDNA was extended during incubation at 68°C for 20 min. The annealing-extension cycle was repeated once more, followed by a final elongation step at 72°C for 10 min. At the beginning of the hot start, a 5 μ L aliquot was mixed with 0.5 μ L of [α ^{32}P]dGTP or [α ^{32}P]dCTP and incorporated. The labeled aliquot was employed at the end of the reaction to measure the cDNA and to calculate the second-strand yield (Carninci and Hayashizaki, 1999).

[0095]

Cloning of cDNA

Second-strand cDNA was treated with proteinase K, extracted with phenol-chloroform and chloroform, and ethanol-precipitated in accordance with standard procedures. The cDNA was then cleaved using 25 U/μ g each of SstI and XhoI (for lung and liver libraries) or *Bam*HI and *Xho*I. Following digestion, cDNA was treated with proteinase K, extracted with phenol-chloroform, and purified on a CL-4B column (Pharmacia). After ethanol precipitation, the cDNA was cloned essentially as described in Carninci and Hayashizaki, 1999.

[0096]

Methodology and Equipment Utilized

Plaque hybridization was conducted with random primer and labeled specific probes in accordance with standard protocols (Sambrook et al. 1989).

Alkali electrophoresis was performed as described (Sambrook et al. 1989). All autoradiography signals were visually displayed using the Bas 2000 imaging system (Fuji).

[0097]

Bacteria were collected with commercially available picking machines (Q-bot and Q-pix; Genetics, UK) and transferred to 384-microwell plates.

Two identical plates were used to prepare plasmid DNA. The plasmid DNA obtained from each of the 384-well plates was divided and grown in four 96-deepwell plates. After overnight growth, plasmids were extracted either manually (Itoh et al. 1997, Nucleic Acids Res 25:1315-1316) or automatically (Itoh et al. 1999, Genome Res. 9:463-470).

[0098]

Sequences are typically run on a RISA sequencing unit (Shimadzu, JAPAN) or using the Perkin Elmer-Applied Biosystems ABI 377 in accordance with standard sequencing methodologies such as described by Hillier et al., 1996, Genome Research, 6:807-828. The sequencing primers are the M13 forward and reverse primers (above described SEQ ID NO: 5 and SEQ ID NO: 6).

[0099]

Example 2

Lung and Liver Tissues

cDNA normalized/subtracted libraries (and minilibraries) were prepared from lung and liver tissues in the same manner as described in Example 1 for brain, with the exception that a primer containing an *Xho*I site (5'$(GA)_8$ACTCGAG$(T)_{16}$VN-3') (SEQ ID NO: 4) and an *Sst*I-containing primer 5'-$(GA)_9$GAGCTCACTAGTTTA-ATTAAATTAATC$_{11}$-3' (SEQ ID NO: 3) were employed for mRNA extracted from lung and liver. The other steps were the same as those described for brain.

[0100]

Example 3

Efficiency of Removal in Driver/Tester Capture

Preparation of an RNA Template

A pBluescript plasmid containing a 5 Kb fragment of reeler cDNA (Hirotsune et al., Nature Genetics, 1995, May, 10(:77-83)) was employed.

From 2.5 μ L of template plasmid DNA (cleaved at the NotI restriction site), RNA was transcribed in vitro using standard conditions described below: 20 μ L of Gibco-BRL 5 X buffer, 5 μ L of rNTPs (10 mM each), 5 μ L of 0.1M DTT, and 20 Units of T7 RNA polymerase at a final volume of 100 μ L. The reaction was conducted by incubation at 37°C for 3 hours. Also, 2 μ L of α-^{32} P-rUTP was added to the reactant to label the RNA.

[0101]

Subsequently, 20 units of RQ1 DNase (Promega) were added to remove trace quantities of template DNA (plasmid) and the sample obtained was incubated at 37° C for 15 min. NaCl was added to the sample to a final concentration of 250 mM. The sample obtained was deproteinated once with phenol (equilibrated with Tris)/chloroform and once with chloroform. Double volume of ethanol RNA was then added to precipitate RNA. After centrifugation for 20 min at 15,000 rpm, the precipitated RNA was separated from the supernatant. The precipitate was washed once with 70 percent ethanol, followed by centrifugation. Finally, the pellet was redissolved in water.

[0102]

Preparation of cDNA (Tester)

cDNA was prepared from the RNA template as specified in the instruction manual of Superscript II (Gibco BRL-Life Technology), with the exception that the primer that was specific to the clone was SK primer (5'CGCTCTAGAACTAGTGGATC3')(SEQ ID NO:7) and that α-32 P dGTP was used to label the first strand for later tracing. Following phenol/chloroform extraction and ethanol precipitation in accordance with standard procedures, the cDNA was then treated with alkali (50 mM NaOH for 30 min) to remove the hydrolized, hybridized RNA and neutralized with 200 mM Tris at pH 7.00. A 20 U quantity of RNase 1 was

added. In the end, the DNA was again extracted with phenol/chloroform and precipitated with ethanol under standard conditions.

[0103]

Biotinylation of RNA

The RNA template was also employed as a driver. Aliquots of 500 ng of RNA were biotinylated with a Biotin-Psoralen kit (Ambion) on ice (corresponding to samples 1-3) or at room temperature (used for samples 4-6) for 30 min (1, 4), 45 min (2, 5) and 60 min (3, 6) (see Fig. 6).

[0104]

Following biotinylation, 50 ng of cDNA (6,000 CPM) and 10 μg of tRNA were added to 150 ng of biotinylated driver (prepared under conditions 1-6, thus 6 tubes) (counting 21,000 CPM),. After standard phenol/chloroform extraction and ethanol precipitation, the samples were redissolved in 5 μL of hybridization buffer (80 percent formamide, 250 mM NaCl, 25 mM Hepes pH 7.5, 5 mM EDTA) and incubated at 42°C overnight (14 hours).

[0105]

Following ethanol precipitation (conducted in the same manner in the other Examples), the samples (6 tubes) were then mixed with streptavidin/magnetic (the subtraction step described in Example 1). The supernatant (unbound) was then precipitated with ethanol under standard conditions with the addition of 4 μg of glycogen to ensure quantitative precipitation, and after resuspension, loaded on a standard RNA/formaldehyde minigel (lanes 1-6). After 1 hour of electrophoresis at 60 V, the gel was dried and exposed with the Bas 2000 image analyzer (Fuji). This shows the efficiency of removal of driver and tester. The (lanes 7-9) side was employed for untreated sample (mRNA/cDNA) corresponding to 10 percent and 2 percent of the starting count of 100. The intensity of the signal indicates the efficiency of removal of the driver/tester mixture.

[0106]

Example 4

Methods of Evaluating the Invention

Reduction in the Frequency of Highly Exmpressed cDNAs

Several normalized/subtracted cDNA libraries were prepared from pancreas tissue in the same manner as described above for brain using RNA drivers and minilibraries derived from the rearrayed nonredundant cDNAs prepared in the above-described Examples to reduce unnecessary resequencing of clones already present.

[0107]

The second-strand cDNA from a standard pancreas cDNA library (without normalization/subtraction) was compared to its normalized/subtracted counterpart (Fig. 3). The normalized/subtracted cDNA was prepared in a single normalization/subtraction step. Normalization was performed at RoT = 10, and subtraction was conducted using a set of minilibraries prepared as set forth above at RoT = 20 (subtraction can be performed at an RoT value of at least up to 500) each of which contained 1,000 to 2,000 main clones mostly abundant from liver, lung, brain, or placreas. The minilibraries were generated by cloning the highly expressed fractions of previously prepared, normalized cDNA libraries. Amplified cDNA minilibraries were then used to prepare the subtracting drivers (as described above). The RoT of the subtracting drivers equaled 1 unit for every 200 clones (e.g., RoT = 5 when 1,000 clones were employed). The average size of normalized, subtracted cDNA was longer than that of non-normalized, non-subtracted cDNA, suggesting that long cDNAs (which migrate more slowly) are expressed more rarely than the shortest cDNAs. In addition, bands corresponding to cDNAs of highly expressed mRNAs are not visible from the normalized-subtracted library.

[0108]

Figure 3 shows an electrophoresis of the non-normalized/non-subtracted one (standard cDNA). A few highly intense bands derived from significantly highly expressed abundant RNAs are visible. By contrast, in the normalized-subtracted cDNA, those bands are no longer visible, suggesting a decrease in the cDNA. Moreover, in the

normalized/subtracted cDNA, the relative intensity of cDNAs corresponding to long mRNAs (>~3 Kb) is greater than in the standard libraries.

Another way of demonstrating the advantages of normalization/subtraction is shown in Fig. 4.

[0109]

First-strand lung cDNA prepared as described above was employed as template. Genes that were abundantly expressed in plaque hybridization functionally corresponding to a normalized cDNA library decreased in the normalized library. When 10,000 plaques of the normalized lung library were screened, elongation factor 1-alpha went from 90 in the reference library to 10 in the normalized library. Carbonyl reductase decreased from about 70 to 3. And uteroglobin was reduced from about 510 to 2 plaques. When the plaques were counted, there were more plaques in the standard cDNA libraries (about 10 times more in the standard library than in the normalized library). These results demonstrated that cDNA with the high expression frequency in the normalized library was much lower than in the control.

[0110]

Example 5

Increasing the Frequency of Discovery of Rarely Expressed Genes

Large-scale library sequencing is the best way to test the concentration of rare ly expressed cDNAs. A number of libraries (Table 1) were prepared from several mouse tissues by the method set forth above and assessed by checking the average size of the cDNA inserts (insert size), sequences passes (Seq.), clusters (Sp.), redundancy (Red.), appearance of new clones (Unique), and presence of full-coding/length cDNA investigated from the percent of sequences having the first ATG codon (coding percent).

[0111]

[Table 1]

Library ID	Development stage / tissue	Normalizing Driver (Rot)	Insert size (kbp)	Seq.	Sp.	Red.	Unique (%)		Coding (%)
18-100	Adult/pancrea	mRNA(5)	1.2	13556	3402	3.98	442	(13.0)	(100.0)
22-000	adult/stomach	(standard)	0.88	1458	488	2.99	42	(8.6)	(82.1)
22-100		mRNA(5)	1.21	4400	1932	2.28	196	(10.1)	(82.1)
22-104		mRNA(5)	1.13	3639	1852	2.11	207	(11.1)	(82.1)
23-000	Adult/tongue	(standard)		1179	556	2.12	36	(6.5)	76.8
23-100		mRNA(5)	1.44	10267	4017	2.56	586	(14.6)	76.8
24-100	ES cell	mRNA(5)	1.77	15226	4495	3.89	485	(10.8)	(88.6)
25-100	Embryo13/liver	mRNA(5)	1.19	5448	1525	3.57	168	(11.0)	92.2
26-000	Embryo10/ whole body	(standard)	1.38	2108	1061	1.99	71	(6.7)	92.3
26-100		mRNA(7.5)	1.32	11267	4722	2.99	582	(12.3)	92.3
28-100	Embryo10+11/ whole body	mRNA(7.5)	1.29	6248	3411	1.83	271	(7.9)	(93.9)
28-104		mRNA(7.5)	1.38	9321	4335	2.15	453	(10.4)	(93.9)
31-000	Embryo/head	(standard)	1.22	488	369	1.32	23	(6.2)	(86.2)
31-100		mRNA(10)	1.55	7838	4229	1.85	494	(11.7)	(86.2)
32-304	Embryo14+17/ head	mRNA(10)	2.5	424	389	1.09	20	(5.1)	(88.2)
38-304	Embryo11/ placenta & extraembryonl c tlaaue	mRNA(10)	1.45	3657	2165	1.69	156	(7.2)	(100.0)
39-304	Embryo13/ whole body	mRNA(10)	2.47	348	319	1.09	22	(6.9)	(90.0)
49-304	Adult/testis	mRNA(10)	2.11	8900	5444	1.63	1214	(22.3)	(95.7)
52-304	Adult/Xlphold	total RNA(3)	2.69	272	256	1.09	15	(5.9)	(100.0)
53-304	Adult/ pltultary grand	total RNA(3)	2.38	8059	4858	1.73	833	(17.9)	(100.0)
54-304	Neonate6/head	mRNA(10)	2.3	2663	2101	1.27	196	(9.3)	(90.0)
57-304	Embryo8/ whole body	(subtracted only)	1.91	19532	7758	2.53	1155	(14.9)	(100.0)
58-304	Adult/thymus	mRNA(10)	3.27	10259	6442	1.59	1100	(17.1)	(80.0)
60-304	Embryo13/ testis	total RNA(5)		11079	6498	1.7	1243	(19.1)	(75.0)
61-304	..Embryo14/ thymus	(subtracted only)	4.13	206	196	1.05	16	(8.2)	(60.0)
62-304	Embryo11/head	mRNA(10)	2.19	2957	2374	1.25	256	(10.8)	(70.0)

[0112]

Assessing the degree of sequence redundancy is the final evaluation of the efficiency of the normalization/subtraction process. Standard libraries (indicated by the reference numbers 22-000, 23-000, 26-000, and 31-000) prepared from an aliquot of the starting cDNA are shown for comparison (Table 1).

[0113]

In one successfully normalized-subtracted cDNA library (for example, library 49-304 from mouse testicular tissue), the redundancy of 3'-end sequences was as low as 1.63 (calculated by dividing the total number of clones sequenced, 8,900, by the number of different clusters, 5,444). From redundancies of less than 2.0 in more than 10,000 to 15,000 3'-end sequences, success could be expected for cDNA libraries from complex tissues (e.g., testes, brain, and thymus).

[0114]

Normalized/subtracted cDNA libraries facilitated efficient and increased recovery of unknown genes. For example, libraries 22-100, 23-100, 26-100, and 31-100 had produced values of new data per sequencing reaction higher than standard library counterparts 22-100, 23-100, 26-100, and 31-100 (Fig. 5). Sequencing several cDNAs from several libraries reveals a decrease in sequence redundancy in the normalized-subtracted libraries as compared to the standard cDNA libraries (Fig.5).

[0115]

In Figure 5, 100 percent new gene discovery corresponds to a redundancy value of 1, 50 percent corresponds to a redundancy value of 2, 25 percent corresponds to a redundancy value of 4, and so forth.

Normalization increases the frequency of new gene discovery relative to standard libraries during a given sequencing test.

[0116]

Example 6

Comparative Example of the Normalization-Subtraction Methods of the Present Invention

The importance of the use of an enzyme cleaving single-strand RNA (driver) bound nonspecifically to cDNA (tester) was checked. Accordingly, sub-libraries prepared by the normalization/subtraction method of the present invention were compared with sub-libraries prepared by the normalization/subtraction method and the step of removing non-specific hybrid.

[0117]

Normalized/subtracted cDNAs prepared in accordance with the first part of Example 1 (through the portion including the normalization/subtraction step) were divided into two subtraction libraries. The first sublibrary was subjected to second strand cDNA synthesis and cloning (that is, without removal of non-specifically bound hybrids), while the second sublibrary was subjected to the RNase 1 treatment (removal of non–specifically bound hybrids) in the same manner as described in Example 1. The mouse tissues prepared are as follows: medulla oblongata for library 63, olfactory brain for library 64, colon for library 90, and cecum for library 91. The data are reported in Table 2.

[0118]

[Table 2]

	Reference Number		Cluster	Unique Clone	p/oUnique Clone	
-RNase I	63-304-R	RISA	2987	252	(8.4)	) 15.4%
+RNase I	63-305-R	RISA	5358	518	(9.7)	
-RNase I	64-304-R	RISA	1258	80	(6.4)	) 81.1%
+RNase I	64-305-R	RISA	6371	742	(11.6)	
-RNase I	90-300-R	RISA	1348	106	(7.9)	) 27.0%
+RNase I	90-302-R	RISA	779	72	(9.2)	
-RNase I	90-304-R	RISA	1066	57	(5.3)	) 62.0%
+RNase I	90-306-R	RISA	1479	127	(8.6)	
-RNase I	91-300-R	RISA	1470	95	(6.5)	) 11.5%
+RNase I	91-302-R	RISA	1786	134	(7.5)	

[0119]

Sublibraries 63-304-R, 64-304-R, 90-300-R, 90-304-R, 91-300-R denote sublibraries that were not treated with RNase 1.

Sublibraries 63-305-R, 64-305-R, 90-302-R, 90-306-R, 91-302-R denote sublibraries treated with RNase 1.

[0120]

The combinations of sublibraries belonging to the same library are: 63-304-R and 63-305-R; 64-304-R and 64-305-R; 90-300-R and 90-302-R; 90-304-R and 90-306-R; and 91-300-R and 91-302-R.

The number of clusters for each sublibrary denotes the number of different clones (that is, clusters) comprised in the sublibrary. Each cluster can comprise one or more clones having the same sequence (that is, the same sequence picked up a number of times).

[0121]

"Unique clones" indicate the number of new clones obtained from a sublibrary cluster that have not been previously sequenced.

The "percentage of unique clones" (for example, the value "8.4" for the sublibrary 63-304-R) denotes the number of unique clones discovered (for example, "252" for sublibrary 63-304-R) divided by the number of clusters ("2987" for sublibrary 63-304-R).

[0122]

In the data of Table 2, all tests of treatment with RNase I yielded high percentages of unique clones (for example, 15.4 percent for sublibraries 63-304-R and 63-305-R). This indicates a number of undiscovered (unique) clones nonspecifically bound to mRNA driver were released from hybrids by the RNase I treatment, recovered, and discovered.

[0123]

The clone sequences were run on a RISA sequencing unit (Shimadzu, JAPAN). M13 forward and reverse primers were employed as sequencing primers. Forward: M13 oligo (5' TGTAAAACGACGGCCAGT 3') (SEQ ID NO: 5); reverse: 1233REV oligo (5' AGCGGATAACAATTTCACACAGGA 3') (SEQ ID NO: 6).

Sequencing was conducted in accordance with a known standard sequencing protocol (Hillier et al., 1996, Genome Res., 6:807-828).

[Sequence listing]

SEQUENCE LISTING

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<120> Method for the preparation of normalized and/or subtracted cDNAs

<130> A05097H/1-12

<160> 7

<170> PatentIn Ver. 2.1

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<223> Description of Artificial Sequence: primer comprising the XhoI restriction site

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<213> Artificial Sequence

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<223> Description of Artificial Sequence: primer comprising the SstI restriction site

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<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: 1233REV reverse primer

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<210> 7

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: SK primer

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[Brief Description of theDrawings]

[Fig. 1] It is a schematic of a suitable normalized and/or subtracted cDNA preparation protocol. A) A general scheme for the preparation of long-strand, full- coding/length single-strand cDNA; B) Representation of a population of different tester cDNAs; C) normalizing drivers (cellular mRNA) and subtracting drivers (run-off transcripts); D) hybridization; E) rare/new cDNAs are used for second strand cDNA preparation (normalized/subtracted cDNA library); F) abundant cDNAs/unnecessary cDNAs are removed and may be used for the preparation of minilibraries to implement subtraction.

[Fig. 2] It shows (on the right side of the drawing) the use of RNase 1 (also referred to as RNase I) able to recognize and cleave RNA (driver) bound non-specifically to cDNA (tester). New and/or rare cDNAs employed in the this method are recovered. In contrast, as shown on the left side of the figure, when no RNase 1 treatment is performed, the new and/or rare tester capable of binding non-specifically to the driver is captured by beads and removed.

[Fig. 3] It shows on the right side an electrophoretic pattern of pancreas cDNA normalization/subtraction performed in one single step compared with a run of pancreas cDNA that has not been normalized/subtracted. This example of normalization/subtraction provides a visual image of the removal of highly abundant full-length cDNAs, which are clearly indicated in the example of cDNA that has not been normalized/subtracted (indicated by arrow).

[Fig. 4] It is plaque hybridization of replicas for the genes EF-1 alpha, carbonyl-reductase and uteroglobin containing a standard (not normalized and not subtracted) length cDNA library (left) or a normalized length cDNA library (right). On the right panel (normalized), the arrow indicates the plaques that have been counted.

The number of plaques (clones) counted for the normalized library is sensitively lower than that for the standard library.

[Fig. 5] The increase in sequencing redundancy (or decrease in new gene discovery) increases sharply in standard cDNA libraries (-000 libraries), but in normalized/subtracted full-length cDNA libraries (-100 libraries) the increase in redundancy is quite slow. New genes (percent) are indicated as singletons (percent) within a given cDNA library.

[Fig. 6] It shows an electrophoresis test for evaluating the efficiency of removal of driver/tester capture using a subtraction method based on the present invention.

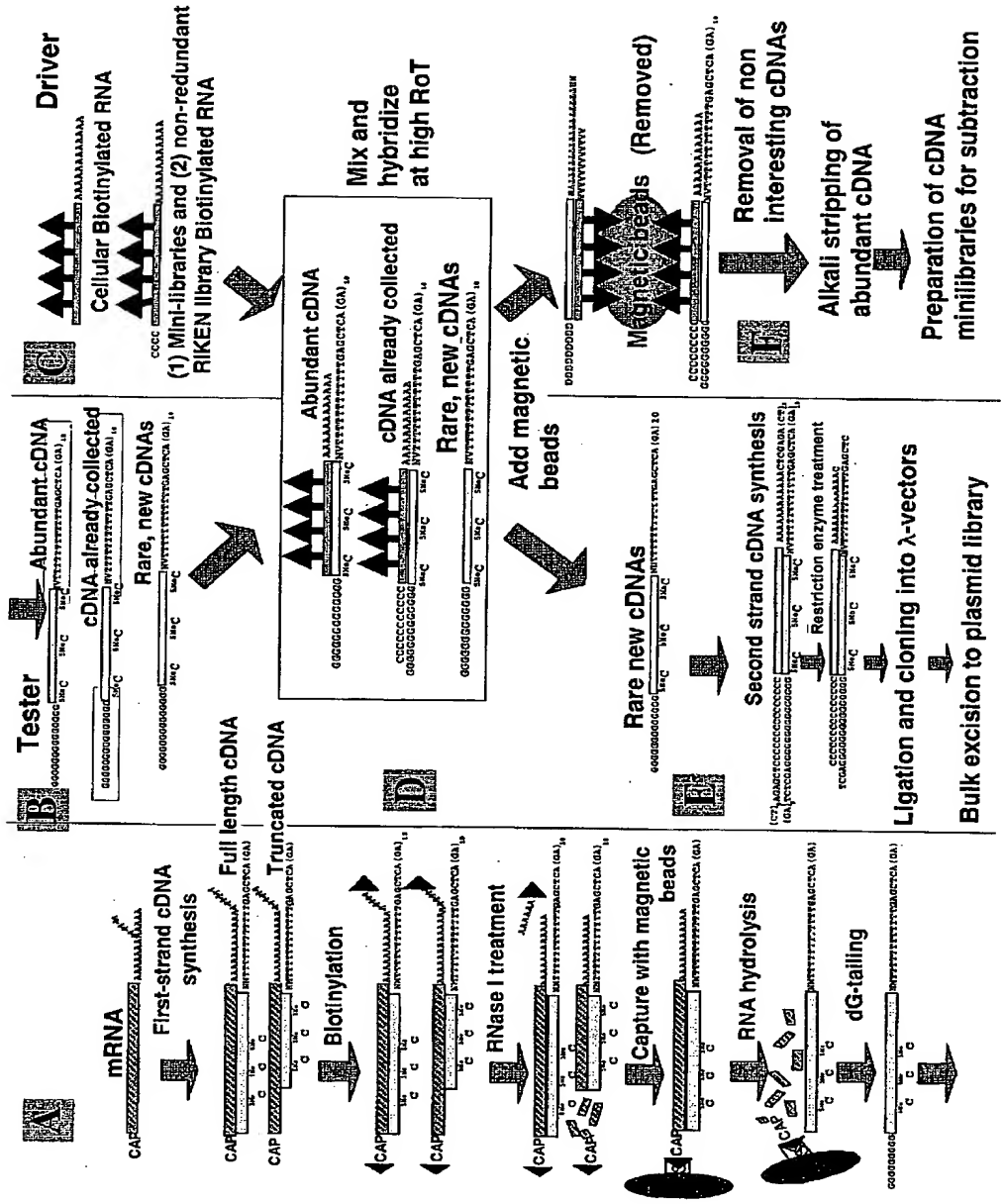
Only one clone (cDNA) tester was used and it was subtracted with a driver made with the same cDNA.

Lanes 1-3 show subtracted cDNA when biotinylation was performed in ice, and lanes 4-6 when performed at room temperature (RT). Lanes 7-9 comprise tester and driver without bead treatment (therefore not removed), showing remaining amounts of 100 percent, 10 percent, and 2 percent, respectively.

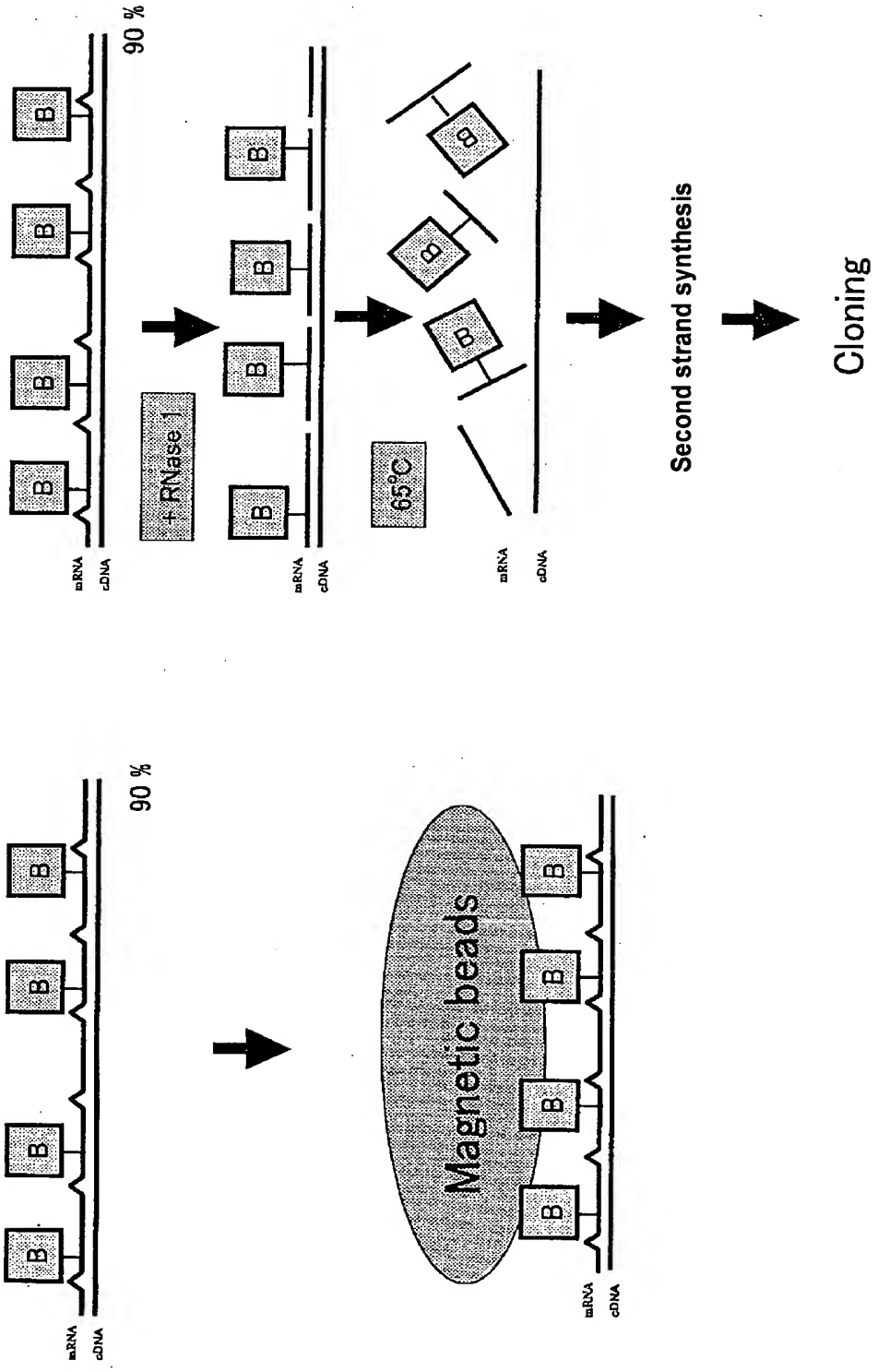
Lane 9 shows that a quantity of 2 percent, even if low, is still evident. In lanes 1-6, it is clear that there is no tester/driver hybrid, indicating that subtraction was almost 100 percent.

[Document] Drawings

[Fig.1]



[Fig.2]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B
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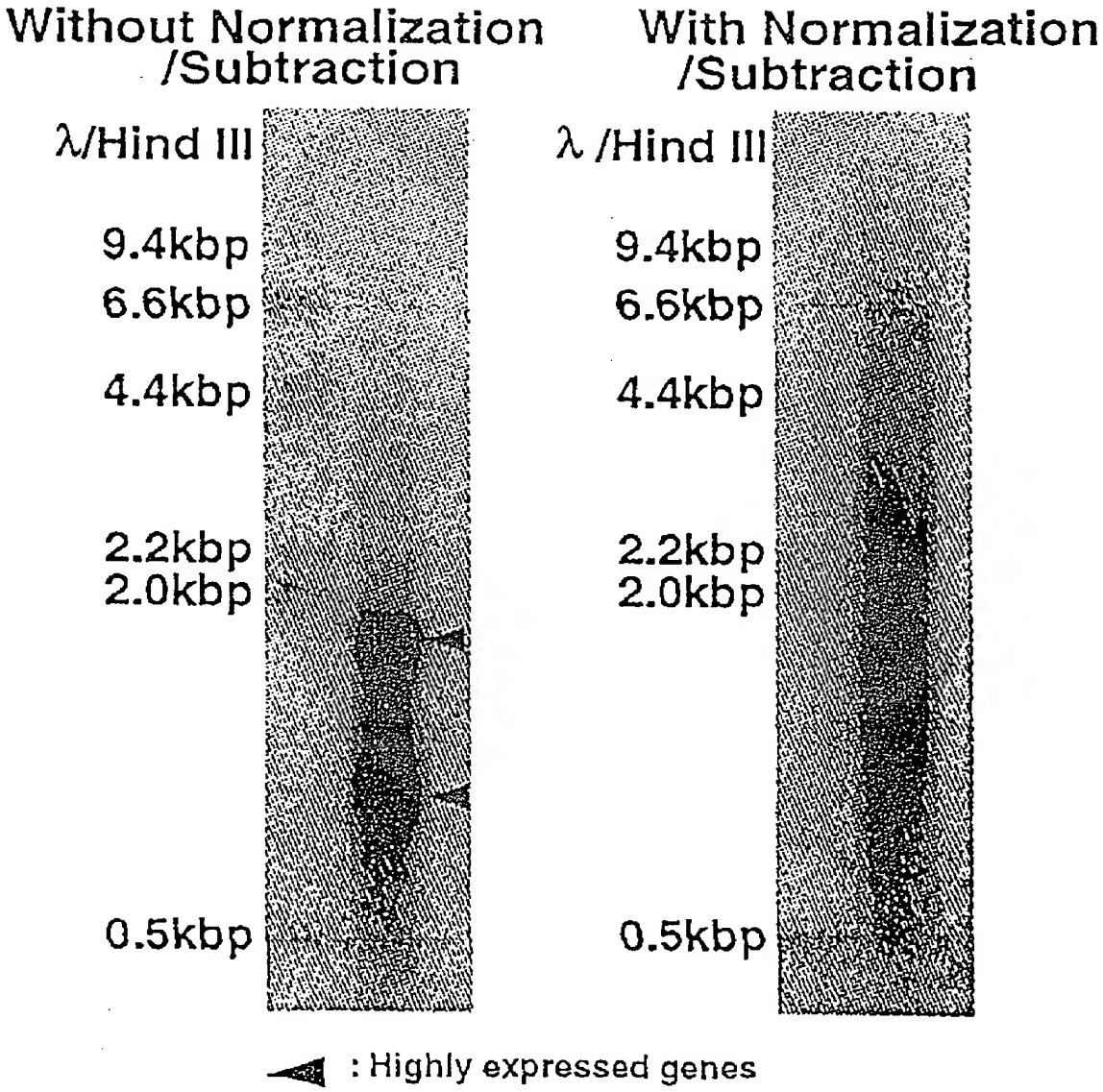
cDNA
+ RNase I
65°C
Second strand synthesis
Cloning
Magnetic beads

[Fig.3]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With Normalization /Subtraction
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λ /Hind III
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2.0kbp
0.5kbp
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4.4kbp
2.2kbp
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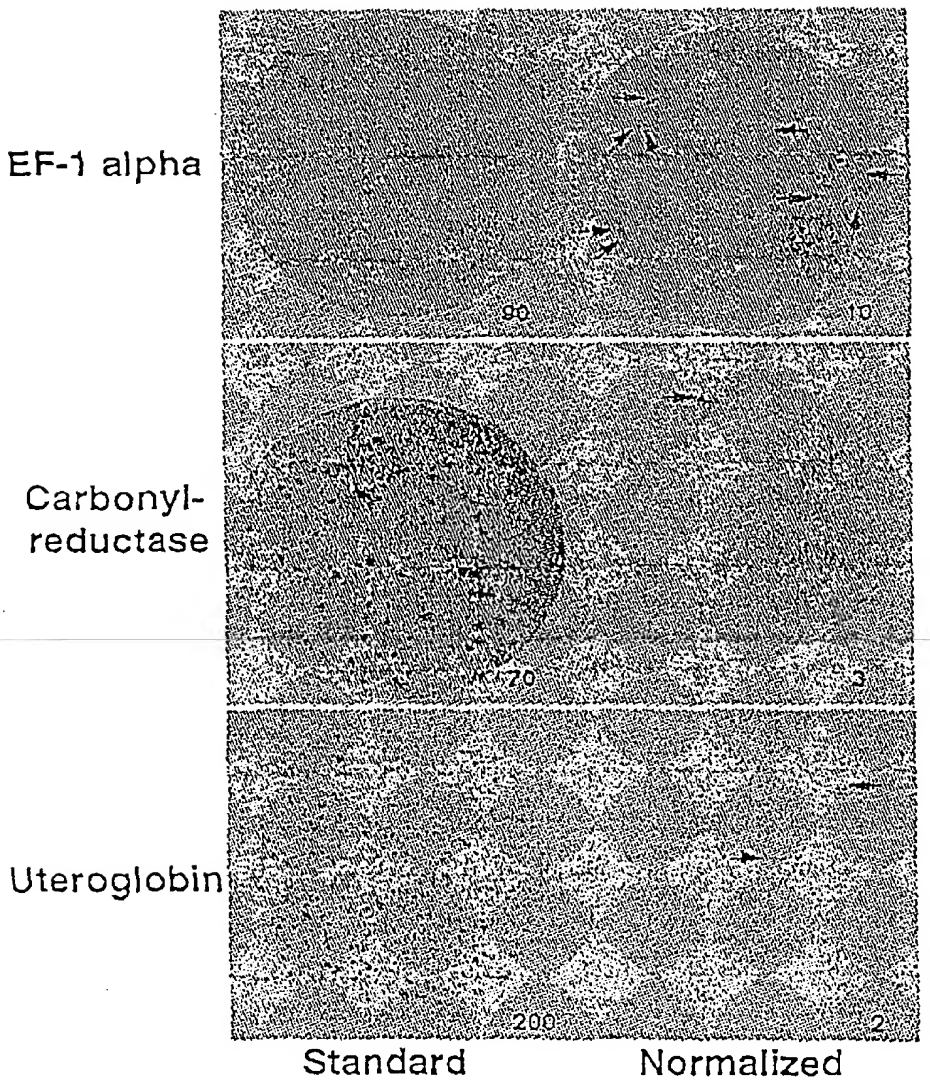
0.5kbp
: Highly expressed genes

[Fig.4]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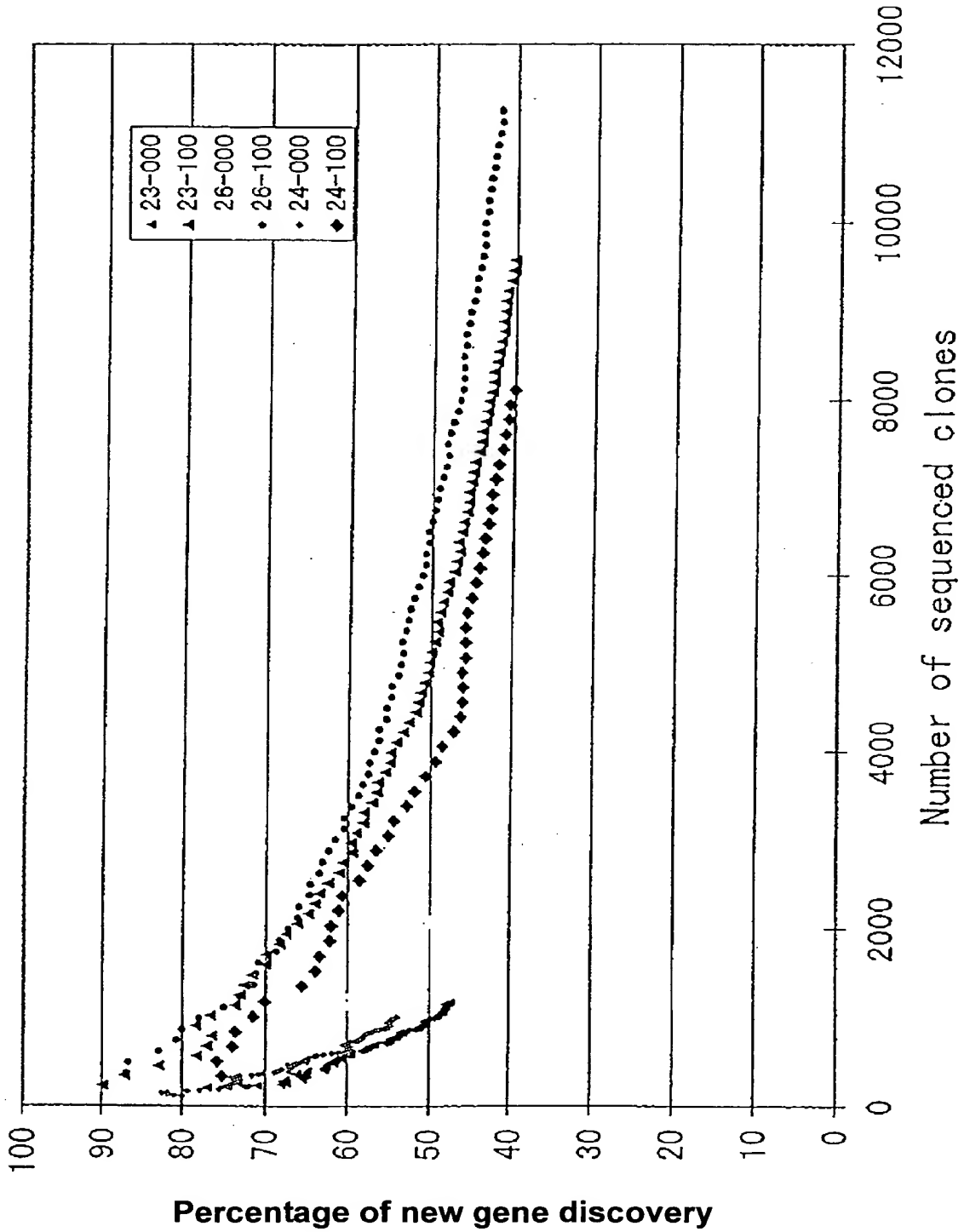
Carbonyl-
reductase
Uteroglobin
90
10
70
3
200
2
Standard
Normalized

[Fig.5]


23-000
23-100
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24-000
24-100
0
2000
4000
6000
8000
10000
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Number of sequenced clones
100
90
80
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50
40
30
20
10
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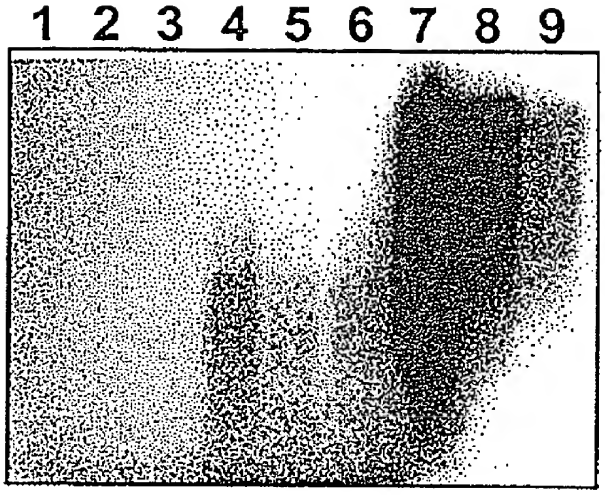
Percentage of new gene discovery

[Fig.6]



[Document] Abstract

[Abstract]

[Problem] To provide a method of efficiently preparing normalized and/or subtracted, long-strand, full-coding/length cDNA library.

[Means for solving problems] A method of preparing normalized and/or subtracted cDNA; a method in which the cDNA that is normalized and/or subtracted is reverse transcript of mRNA in the form of uncloned cDNA (cDNA tester); it comprises (a) a step of preparing cDNA testers; (b) a step of preparing normalization and/or subtraction RNA drivers; (c) a step conducted by mixing the normalization/subtraction RNA driver with said cDNA tester; (d) a step comprising addition of an enzyme capable of cleaving single strand sites on RNA drivers non-specifically bound to cDNA tester; (e) a step of removing the single strand RNA driver cleaved from the tester and removing tester/driver hybrids; and (f) a step of recovering the normalized and/or subtracted cDNA.

[Selected figure] Fig. 1